THE FERTILIZATION REACTION IN ECHINARACHNIUS PARMA.

II. The Role of Fertilizin in Straight and Cross Fertilization.

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Contents.

I. Introduction 12
II. Sperm Agglutination and its Significance in *Echinarachnius* 12
A. Sperm Agglutinin Production in *Echinarachnius* 13
1. Quantitative Method for Determining Fertilizin Production by Ova 13
a. Variations in Fertilizin Production by Ova 14
b. Fertilizin Production by Washed Eggs 15
2. Source of Fertilizin 16
a. Comparison of Ripe Eggs With and Without Jelly 16
b. Study of Immature Eggs 17
c. Study of the Perivisceral Fluid 18
B. Fertilizin Production as an Index of Fertilization Capacity 18
1. Comparison of "Ovary Eggs" and "Dry Eggs" 19
2. Fertilization capacity of Washed Eggs 20
3. Eggs Normally Resistant to Fertilization 21
4. Fertilizin Production after Membrane Formation 22
a. Eggs with Fertilization Membranes 22
b. Eggs with Butyric Acid Membranes 24
C. Summary of Part II 24
III. Cross Fertilization and Agglutination in *Echinarachnius* and *Arbacia* 25
A. Reciprocal Cross Fertilization between *Echinarachnius* and *Arbacia*. 25
1. *Arbacia* ♀ × *Echinarachnius* ♂ 26
2. *Echinarachnius* ♀ × *Arbacia* ♂ 27
B. Hetero-agglutination in *Echinarachnius* and *Arbacia* 29
1. Effect of *Echinarachnius* Egg-water on *Arbacia* Sperm 29
2. Effect of *Arbacia* Secretion on *Echinarachnius* Sperm 30
a. Agglutinative Power of *Arbacia* Egg-water on *Echinarachnius* Sperm after Successive Dilution 30
b. Hetero-agglutinin Production of Washed *Arbacia* Eggs .. 31
c. Precipitation of Hetero-agglutinin by *Echinarachnius* Sperm 32
d. Agglutinative Action of *Arbacia* Blood on *Echinarachnius* Sperm 32
e. Microscopic Appearance of *Echinarachnius* Sperm Agglutinated by *Arbacia* Egg-water 33
C. Summary of Part III 33
IV. Discussion 34
V. Literature Cited 37

I. Introduction.

The behavior of the sperm cells with reference to egg-extractives and the significance of this for the mechanism of fertilization has been analyzed in detail only in *Arbacia* and in *Nereis* (Lillie, '13, '14). It is true that the agglutination reaction had been previously studied by various workers, but the rôle of this phenomenon in fertilization as a specific reaction was not suggested; and it is on this specificity that the fertilizin theory of Lillie in large part rests; that is, that the egg alone secretes the sperm-agglutinating substance and that this substance is necessary for fertilization. It seemed desirable by study of this agglutinating reaction to determine its rôle in fertilization in some other form; this has been done in *Echinarachnius parma*. The present paper is a contribution to the analysis of the rôle of the sperm-agglutinating substance as revealed by a study of its behavior in straight and in cross fertilization. The results conform with those of Lillie, the data on cross fertilization seemingly at variance actually giving support to the view that the agglutination reaction is specific. The sperm agglutinin of *Echinarachnius* is, therefore, identical in behavior with the fertilizin of *Arbacia*.

Although observations on *Echinarachnius* were made during 1914, and 1915, all experiments here reported except those cited on page 22 are from those made, at the Marine Biological Laboratory at Woods Hole, Mass., during the season of 1917, beginning early in June. The experiments fall into two groups: those on iso-agglutination and straight fertilization in *Echnarachnius* (Part II.) and those on hetero-agglutination and cross fertilization with *Arbacia* (Part III.).

II. Sperm Agglutination and its Significance in Echinarachnius.

The mere fact that sperm are agglutinated by substances dissolved in sea-water is of no special significance for us since many substances will accomplish this; among others, alkalis, polyvalent salts (Gray), and snake venom (Noguchi); but the agglutinations by these agents are distinctly toxic and we must, therefore, at the outset exclude them from consideration. They

must in no wise be confused with agglutination produced by egg secretions. What is significant is that the agglutinin produced by eggs behaves in a characteristic fashion, may be quantitatively studied, and is a necessary part of the fertilization reaction.

A. *Sperm Agglutinin Production in Echinarachnius.*

Eggs of *Echinarachnius* charge sea-water with a substance that agglutinates *Echinarachnius* sperm. If a drop of this charged sea-water be added to a sperm suspension the sperm rapidly form clumps. The eggs give off this agglutinating substance in the absence of the jelly hulls enclosing them. Stale eggs give off relatively little and immature eggs none, of the agglutinin.

1. *Quantitative Method for Determining Agglutinin Production by Ova.*

By the method of successive dilutions of the sea-water taken from above *Echinarachnius* eggs after they have settled one may obtain quantitative estimates of the agglutinating power of the egg-secretion. This method, used by Lillie on *Arbacia*, briefly is as follows: A series of successive dilutions of the sea-water above *Echinarachnius* eggs is made, each dilution being one half the preceding. Usually I started with a 1/100 dilution of the agglutinin charged sea-water; a half dilution gives 1/200. Thus the series 1/100, 1/200, 1/400, 1/800, etc., is made. As noted below, series of 1/2, 1/4, 1/8, 1/16, etc., were likewise employed, so too, 1/10, 1/20, 1/40, etc. The agglutinating power of these dilutions is tested on sperm suspensions until the reaction comes negative. The last dilution to give a positive reaction rates the power of the agglutinin.

To observe the agglutination, drops of sperm suspension are mounted on a slide the cover slip of which is supported by glass rods, the slide being placed on the stage of the microscope in focus under low power. Into this sperm suspension, by means of a capillary pipette attached to a rubber tube, is blown a drop of the sea-water from the eggs. This microscopic method, as will be shown beyond, is indispensable for a comparative study of the agglutination reaction, or indeed for accurate quantitative determinations.

Agglutination may also be observed by adding two or three drops

of milky sperm suspension to two cubic centimeters of strong egg-water.[1] The sperm form clumps of such size that they are clearly visible to the naked eye, the sea-water between the masses remaining clear, so sharp is the reaction. This method suffices merely to indicate the *fact* of agglutination, and this only in high concentration. It was employed but seldom in these experiments as will be noted in the following pages. Indeed, by this method alone the critical points in this whole work would scarcely have been determined.

The methods for handling eggs and sperm are important since the state of the egg or sperm suspension conditions the experiment. To obtain eggs relatively free of debris, a mass of ovaries in two or three times their bulk of sea-water is strained through cheese cloth. Or, the animals may be rapidly opened and placed aboral side down in dry watch glasses and dry eggs collected as they exude from the genital pores. Also, eggs may be got as they are normally shed by animals in separate glasses. Sperm is collected from those males that have been opened, dried and placed aboral side down in dry watch glasses, and that soon shed sperm being pipetted off as needed. The sperm suspensions mentioned below, unless specifically noted to the contrary, were always one per cent. suspensions made by the addition of one drop of dry sperm to ninety-nine drops of sea-water. It is imperative to make up the suspension only as needed, for sperm reactions show wide variations depending upon freshness and upon concentration which likewise is a factor in ageing; the less concentrated suspensions deteriorate faster than more concentrated. Finally. everything was kept scrupulously clean, and all means taken to prevent contamination which might easily have invalidated whole series of experiments.

(*a*) *Variations in Fertilizin Production.*—Eggs vary in their capacity for producing fertilizin. In part this variation may be due to the fact that some suspensions do not consist wholly of eggs but may contain bits of tissue, etc.; such a suspension will be inferior to another of equal concentration but containing eggs only. However, this does not fully explain the variation, for it

[1] By egg water is meant sea-water taken from above eggs that have stood for a time.

is a simple matter by exercising care to procure, as mentioned above, from ovaries in sea-water clean egg suspensions entirely free from bits of tissue; these eggs as a class (which to avoid circumlocution I designate "ovary eggs") show wide variations and are inferior to dry or shed eggs, *i. e.*, those deposited by animals in clean dry dishes. Except for eggs mentioned on page (21) as normally resistant to fertilization, dry eggs produce more agglutinin than "ovary eggs" but show less variation.

The following observations from a large body of recorded data may now be cited.

1. July 11, 9:30 A.M.: To 1/2 c.c. of dry eggs sea-water was added to the 10-c.c. mark. At 10:10 7 c.c. of the supernatant sea-water was withdrawn and filtered. Tested with freshly prepared 1 per cent. *Echinarachnius* sperm, this withdrawn agglutinin charged sea-water was negative at 1/3,200 dilution and gave an 8 second reaction at 1/1,600.

2. July 11, 9:40 A.M.: Ovaries of 8 females in 20 c.c. of sea-water strained. Sea-water added to 100 c.c. Eggs settled at the 7 c.c. mark. At 10:20, 90 c.c. of supernatant sea-water decanted and filtered, which when tested on sperm came negative at 1/400 and gave a 6 second reaction at 1/200.

3. July 10, 10:30 A.M.: To 1.5 c.c. of dry eggs 8.5 c.c. of sea-water were added. 11:00 A.M. 8 c.c. of supernatant sea-water decanted and filtered, 8 c.c. being added to the eggs. 11:00 A.M. to 12:10 P.M., supernatant water of the *second washing* gave a 6-second reaction at 1/12,800 dilution.

Comparison of (1) and (2) shows that a 5 per cent. suspension of dry eggs yielded fertilizin of 1/1,600 power while a 7 per cent. suspension of ovary eggs contained fertilizin of but 1/200 power. Also, (3) shows us that after a *second washing* a 15 per cent. dry egg suspension gave fertilizin rated at 1/12,800 power. Without exception, the most powerful fertilizin suspensions were from shed eggs. These results are typical of a large number of observations.

(*b*) *Fertilizin Production by Washed Eggs.*—Lillie has shown that in *Arbacia* it is practically impossible by washing to remove all the fertilizin; as long as the egg lives it produces the agglutinating substance. The egg of *Echinarachnius* is not so hardy as that of *Arbacia;* becoming physiologically inert after remaining in sea-water for a time, it ceases to give off fertilizin though far from dead. This cessation is gradual, with each successive washing the fertilizin produced being less. Two experiments are cited.

1. July 2, 10:00 A.M.: Eggs from 4 females washed 5 times in 250 c.c. of sea-water at each washing. 2 c.c. of eggs after the fifth washing. At 12:00 M. 210 c.c. of supernatant water withdrawn and filtered, 210 c.c. of sea-water being added to the eggs. A sample of this removed at 1:30 P.M. on testing gave at 1/1,600 dilution a 10 second reaction. This is equal to morning tests. Washed 5 times during the afternoon by removing 210, 217, 203, 190, and 205 c.c. of water and adding in each case the quantity withdrawn. Tested at 8:30 P.M., supernatant sea-water gave an 8-second reaction at 1/1,600. July 3, 9:00 A.M. 210 c.c. of supernatant sea-water removed, 210 c.c. added. Tested during the day to 1:30 P.M. negative.

2. July 3, 9:10: Ovaries of 5 females in sea-water strained. Sea-water added to 100-c.c. mark. Washings (75 c.c. of water removed each time) as follows: 9:30, 9:34, 9:38, 10:05, 10:12 during the morning and at 1:50, 2:30 and 2:38 during the afternoon. Tested at 9:40, 10:50 and 1:30 gave 10 second reactions at 1/100. At 2:40, 8-second reaction on 1/50 dilution. 2:55 P.M. 75 c.c. of supernatant water removed, 75 c.c. added. 5 P.M., reaction faintly positive at 1/50. July 4, 10:30 A.M. 75 c.c. removed, 75 c.c. added. 11:10 A.M. negative when tested.

2. *Source of Fertilizin.*

In *Arbacia* only mature eggs produce fertilizin; immature eggs are incapable of secreting the agglutinating substance; other tissues do not elaborate it, nor does it occur in the perivisceral fluid (Lillie, '13, '14). In *Echinarachnius* mature eggs alone with or without jelly yield fertilizin to the sea-water. Neither the immature egg nor the body-fluid is capable of charging sea-water with sperm agglutinin.

(*a*) *Comparison of Ripe Eggs With and Without Jelly.*—Though the jelly is loaded with fertilizin, it does not form the fertilizin for jelly-free eggs are capable of charging the sea-water with the sperm agglutinating substance. The jelly hulls enclosing *Echinarachnius* eggs may be removed by shaking or by treatment with acid. The method of removing the jelly by shaking demands care since agitation may cause injury to the eggs. If shaking brings about the binding of fertilizin we should get a cessation of fertilizin production. This should not be attributed to mere loss of jelly, though it might be so interpreted by some. Again, it might be argued that the use of acid to remove the jelly might injure the egg; this objection could be raised only in the event that fertilizin is no longer produced by the egg after acid treatment, which is at least remotely possible. Certainly, it would be gratuitous to argue that if the fertilizin continues to be produced after acid treatment the acid carries the fertilizin from jelly to egg! In short, after shaking or after acid treatment

the cessation of fertilizin production would not prove that the jelly forms fertilizin. However, after both acid treatment and gentle shaking the eggs still produce fertilizin.

Echinarachnius eggs may be shed without jelly. Often in a given lot of eggs some will be found without jelly, and on three occasions during the summer I observed eggs shed *not one of which had a jelly covering.* These eggs proved to be normal in every respect; secreted fertilizin, were capable of fertilization, and of normal development.

I cite three experiments:

1. One c.c. of dry eggs was placed in 50 c.c. of sea-water plus 3 c.c. of *n*/10 HCl. Gently stirred to prevent clumping and washed in four changes of sea-water. Left in 50 c.c. of sea-water. Examination revealed absence of jelly in most eggs. Tested after one hour, the egg water gave an 8-second reaction at 1/200 dilution.

2. One c.c. of eggs was gently shaken five or six times in 10 c.c. of sea-water. Most of the eggs lost their jelly. The eggs were washed 5 times in 10 c.c. of sea-water and left finally in the cylinder in 10 c.c. of clean sea-water. On settling the eggs were at the 0.5-c.c. mark; tested, the supernatant sea-water gave at 1/400 dilution a 6-second reaction.

3. One c.c. of dry eggs shed without jelly was placed in 24 c.c. of sea-water. Tested an hour later the egg-water gave at 1/1,600 dilution a 6 second reaction. After a sixth washing in 3 hours these eggs produced agglutinin giving an 8-second reaction at 1/800 dilution.

Whatever may be the interpretation of the experiments with eggs freed of jelly through shaking or acid treatment, the conclusion drawn from the results with these eggs lacking jelly when shed is inescapable: in *Echinarachnius* as in *Arbacia* the fertilizin is secreted by the egg, however much the jelly may be loaded with it.

(*b*) *Study of Immature Eggs.*—During the season one may always obtain immature sand-dollars. Indeed one gets the impression that there is a rhythm in the breeding season. Loeb's observations ('15) would suggest that this is true of the California urchin, while Tennent[1] has noted the phenomenon which suggests lunar periodicity in the breeding of the Atlantic urchins. I was able, therefore, early in the season to determine quite definitely that immature *Echinarachnius* eggs do not give off fertilizin. Thus, on July 3, immature eggs from three females

[1] Dr. Tennent also tells me that in Greece the gonads of urchins used as food appear on the market only during certain moon phases; at other times the animals are spent.

were in turn tested. The supernatant sea-water from none during ten hours gave an agglutination reaction. Ovarian tissue, likewise, procured after removing the eggs by straining, does not produce a sperm agglutinin. The fully matured egg alone with or without jelly elaborates fertilizin.

(*c*) *Study of Perivisceral Fluid.*—Finally, repeated trials with with perivisceral fluid from mature or immature females never gave agglutination. Thus, at 2:40 P.M., July 3, drops of 1 per cent. *Echinarachnius* sperm suspension placed on slides when tested with perivisceral fluid from three females showed no agglutination. Each of the three samples of fluid was then diluted with clean sea-water 1/2, 1/4, 1/8, 1/16. The reaction with fresh sperm suspension in all cases was negative.

To sum up, we may say that mature *Echinarachnius* eggs in sea-water give off a substance that agglutinates *Echinarachnius* sperm; this substance the eggs will form in the absence of their jelly hulls. Immature eggs do not secrete the agglutinin nor does the ovarian tissue or perivisceral fluid.

The agglutinin of *Echinarachnius* is more powerful than that of *Arbacia*, as can be readily gleaned from a study of Lillie's work. An equal quantity of *Echinarachnius* eggs will give off more fertilizin in a given length of time than *Arbacia* eggs, but *Echinarachnius* eggs will cease production before *Arbacia* eggs. This is doubtless correlated with the greater irritability of *Echinarachnius* egg as evidenced both by its susceptibility to mechanical agitation and its earlier loss of fertilizing power.

It can be readily shown that this loss of fertilizing power is gradual and farther that it runs parallel with lessening fertilizin production.

B. *Fertilizin Production as an Index of Fertilization Capacity.*

While it is true that only mature eggs are capable of producing the agglutinin, this capacity varies between wide limits. One cannot foretell, therefore, the agglutinin production of a given lot of eggs in a given quantity of sea-water. Often eggs which on microscopic examination are found to be fully matured yield no agglutinating substance. Since, as shown above, immature eggs do not secrete fertilizin nor does the jelly form it, the secre-

tion must occur after the formation of the polar bodies. Fertilizin production, therefore, may be regarded as the sign of *physiological maturity*. This is borne out by a study of the parallel between fertilization capacity and fertilizin production. Eggs incapable of fertilization either have produced no fertilizin, unripe eggs, or have lost fertilizin, stale (washed) eggs.

1. *Ovary Eggs and Dry Eggs.*

As has been mentioned above, by cutting up ovaries in seawater and straining the mass one may procure fairly clean egg suspensions free from bits of tissue. These are the "*ovary eggs.*" Dry or shed eggs are those deposited by the animals in clean dry dishes.

"*Ovary Eggs.*"—Different lots of "ovary eggs" vary greatly with respect to the agglutinin production, as repeated observations showed. A ten per cent. suspension made up of eggs from one female may give a positive reaction on 1/6,400 dilution while another ten per cent. suspension from another female may show little or even no agglutinative power. The capacity for fertilization corresponds so that following insemination ninety per cent. or more of the eggs with strong agglutination action cleave; very few of the eggs of no agglutinative action cleave. This is due to the fact that by cutting up the ovaries one obtains eggs of different degrees of ripeness. One lot from a given female may consist of fully ripened eggs while another lot contain scarcely any ripe eggs despite their appearance. If after washing the same pieces of ovaries several times, one collects eggs after each washing, the percentage of fertilization drops with each washing; the first lot of eggs is made up doubtless of those ready to be shed and so are fully ripened; successive lots are less mature. Moreover, dishes of "ovary eggs" usually contain some ovocytes. Without exception among these eggs those that fail to develop, give off no agglutinin; but those that have great capacity for fertilization show high agglutinating power.

"*Dry Eggs.*"—Dry or shed eggs are perfectly ripe, of high agglutination action, giving practically one hundred per cent. cleavage, beautiful large gastrulæ, and vigorous hardy plutei. The difference between these and "ovary eggs" is striking. Apparently when eggs are shed they are fully ready for fertiliza-

tion. Some years ago I observed similar behavior in *Asterias*. Eggs cut from ovaries may or may not fertilize: if they do cleave, subsequent development may be uncertain. Eggs naturally shed on the other hand, *may be inseminated at once*, fertilize easily, and develop perfectly, giving rise to larger and more vigorous larvæ. It would be a mistake to reach conclusions concerning the behavior of eggs and sperm unless these products have also been studied as they are normally shed. And I am disposed to believe that a great deal of the variability of eggs, so called physiological condition met with by workers, is due to the use of eggs not perfectly ripe. Dry eggs give powerful agglutinative suspensions.

2. *Washed Eggs.*

It is generally known that eggs lose their capacity for fertilization through remaining in sea-water, though the resistance to sea-water may vary within very wide limits depending upon the species of egg. Thus, the *Platynereis* egg can no longer be fertilized after six to ten seconds in sea-water, whereas the *Arbacia* egg may still be fertilized after several hours. In *Echinarachnius* the power to withstand sea-water varies with the kind of egg ("ovary egg" or dry), the time of season, the temperature, amount of mechanical shock, amount of sea-water used, etc. The most important factor is the condition of the egg since the most perfectly ripened eggs, *i. e.*, shed eggs, lose fertilization capacity earliest. There is an optimum period for fertilization and this gradually passes off as the eggs lie in sea-water. Eggs may lose their fertilization capacity in from two to fifteen hours; washing the eggs repeatedly will hasten this loss. Thus,

(*a*) 1 c.c. of eggs, samples of which when tested were fertilizable, was suspended in 99 c.c. of sea-water at 4:30 P.M., July 6. The supernatant sea-water gave on 1/800 dilution an 8-second reaction. Next day at 9 A.M. these eggs were unfertilizable before or after washing in 100 c.c. of sea-water. The superantant sea-water was negative to *Echinarachnius* sperm when tested throughout the day.

(*b*) The following is typical of a large number of experiments. On July 6 at 8 A.M., 2 c.c. of eggs were collected from three fine females washed in 5 c.c. of sea-water and strained. They were

washed seven times, agglutination tests and inseminations being made at each washing. The table gives the data.

EGGS FROM OVARIES OF THREE FEMALES.

Time.	Washings. No.	Agglutination Reaction.			Per Cent. Cleavage.
		C.c. of Sea-water Used.	Time in Seconds.	Dilution.	
8:30	1	100	8	1/1600	90
9:00	2	200	8	1/100	90
9:15	3	100	6	1/800	85
9:40	4	200	6	1/400	80
10:00	5	200	6	1/200	70
12:00	6	75	4	1/50	10
12:10	7	85	4		10

Began with 4 c.c. of eggs, ended with 2 c.c.

Two points are worthy of note respecting this table: First, the amount of water used in washing was unfortunately not the same throughout; and second, which is unavoidable, the quantity of eggs diminished with each washing. These two factors suffice to account for apparent irregularities in per cent. of cleavage and in agglutination. Thus, nos. 1 and 2 give the same per cent. of cleavage, whereas in no. 2 the agglutination reaction is but half as strong, but it is also true that twice as much sea-water was used in no. 2. The time between the washings is likewise irregular; this presumably would affect the amount of agglutinin present in the sea-water. However, the end result is unmistakable: low fertilization and low agglutination reaction are parallel. Since freshly made sperm suspensions were used for each insemination, the only other possible explanation is that the eggs die in large numbers. This is rather hazardous if not gratuitous since the eggs may make abortive attempts at development.

3. *Eggs Normally Resistant to Fertilization.*

On July 15 I procured 7 c.c. of dry eggs which were placed in 65 c.c. of sea-water. Also, by placing ovaries in sea-water for a few minutes before straining I obtained 3 c.c. of eggs which seemed to be unusually fine. Eggs from both lots were inseminated for preservation of a normal series. To my chagrin relatively few of these eggs segmented. July 16 gave similar results. Not until later did experiment reveal the cause of this failure to

segment. The observation of July 20 may serve as a typical case.

At 10:10 A.M., dry *Echinarachnius* eggs were placed in sea-water; total bulk of eggs plus sea-water equalling 10 c.c. (*Lot A.*)

10:20 A.M., 10 c.c. of ovaries plus 90 c.c. of sea-water were placed in a cylinder which was gently inverted three times. (*Lot B.*)

10:50 A.M., 7 c.c. of sea-water removed from eggs of *Lot A* filtered and diluted as follows: 1/10, 1/20, 1/40, 1/80, 1/160. 7 c.c. of fresh sea-water added to eggs. Trials with sperm of two males gave a 6-second reaction at 1/20.

11:20 A.M., 6 c.c. of supernatant sea-water removed and filtered, dilutions being made as before. (6 c.c. of fresh sea-water added to eggs.) 4-(?)-second reaction at 1/10 dilution; very faintly positive.

11:50 A.M., 5 c.c. of supernatant sea-water removed and filtered with dilutions as before, 6-second reaction at 1/10. Eggs inseminated 3:00. These normally fertilized eggs show very few cleavages. *Lot B* gave comparable results.

If this were a long series of washings extending over two or three days, one might suggest that these eggs no longer fertilize or give the agglutination test because they are dead. One could scarcely have the hardihood, though, to venture that the eggs die after remaining in sea-water some ninety minutes. Rather these eggs fail to fertilize, as was discovered during the next two days because of some membrane condition, for on the addition of ether to the sea-water, eggs, of which but 1 per cent. showed cleavage in normal sea-water, gave 90 per cent. cleavage and normal larvæ. I might designate these eggs as over-ripe following R. S. Lillie's usage since they behave so much like over-ripe *Asterias* eggs. The cleavage percentage of these eggs is always low and their agglutinin production feeble. Unfortunately, I failed to study the agglutinative power of these ether-treated eggs.

4. *Fertilizin Production After Membrane Formation.*

Observations on eggs with membranes—which were not made before the season of 1918—show that after insemination or successful butyric acid treatment fertilizin production ceases.

(*a*) *Eggs with Fertilization Membranes.*—Lillie found ('14, pp. 553–557) that in *Arbacia* fertilized eggs with jelly removed by shaking ceased to give the fertilizin test after five washings (extending over a period of one hour and forty-three minutes), the last washing being made at two hours and twenty-three minutes after insemination; while an equal quantity of eggs of

the same inseminated lot *with* jelly still gave a test of over seventy-five seconds. When these latter came negative is not stated. From this and duplicate experiments Lillie concluded that after fertilization the egg no longer produces fertilizin; the positive tests with jelly-covered eggs show merely that the jelly is saturated with the secretion. I have repeated this experiment with *Echinarachnius* eggs and have also made some others slightly different. My findings agree with those of Lillie.

An experiment of August 1 is typical of a number:

9:15 A.M. Two tubes (*A* and *B*) each with 0.5 c.c. of eggs from the same female plus 9.5 c.c. of sea-water; 9:16 A.M. inseminated; 9:18 A.M. all eggs have membranes; 9:20 A.M. tube *B* gently shaken to remove jelly; 9:23 A.M. jelly off every single egg, membranes intact. By means of a capillary pipette attached to a rubber tube supernatant sea-water carefully removed from each tube as follows: 9:35, 9:45, 10:00, 10:16, 10:31, 11:00 and 11:13 A.M.; in each case 8 c.c. being removed and 8 c.c. of fresh sea water added. At 11:05 A.M. at 1/1 dilution *B* is slightly positive (?); at 11:15 A.M. and thereafter *B* is negative at 1/1 dilution. *A* at 1/8 dilution gave an 8-second reaction. 99 + per cent. of the eggs in both lots developed normally.

Thus in two hours after insemination (seven washings) the tests on jelly-free eggs came absolutely negative. (*Vide supra*, Lillie's results.)

The data on some of these experiments seemed to indicate that the fertilizin is not lost in any given time but rather after a certain amount of washing. If this be true, a certain amount of water ought to remove from a given lot of eggs the free fertilizin practically at once. The following experiments show this:

Aug. 2, 9:40 A.M. Two cylinders (*A* and *B*) each with 0.5 c.c. of eggs from the same female plus 9.5 c.c. of sea water. Both inseminated. 9:43 A.M. *B* shaken. Eggs have 100 per cent. membranes in both *A* and *B*; none have jelly in *B*.

9:50 A.M. Lots *A* and *B* each in 250 c.c. of sea-water.

9:53 A.M. *A* is positive at 1/2 dilution; *B* is negative at 1/1 dilution, never again positive.

Aug. 3, 9:58 A.M. 2.5 c.c. of eggs from one female plus 7.5 c.c. of sea-water inseminated and divided into two equal lots—*A* and *B*. *B* shaken. *A* and *B* each added to 250 c.c. of sea-water. 10:05 A.M. *B* positive at 1/1 dilution. 10:07 A.M. 190 c.c. of sea-water removed from *A*, 200 c.c. of sea-water removed from *B*. 190 c.c. and 200 c.c. of sea-water added to *A* and *B* respectively. 10:25 A.M. *A* barely positive at 1/8 dilution; *B* is negative at 1/1.

These observations bear out findings of June: while making experiments with shaken eggs I tested fertilized jelly-free and membrane-free eggs and never obtained a positive test. Evi-

dently, Lillie's assumption "that all free fertilizin is fixed *at the moment of* fertilization or membrane formation" is correct.

(*b*) *Eggs with Butyric Acid Membranes.*—If eggs be successfully treated with butyric acid (optimum exposure to optimum concentration of butyric acid in sea-water) they form membranes. In highly successful cases one may obtain one hundred per cent. membranes. These eggs behave as do eggs with fertilization membranes. If washed by repeated removal of the supernatant sea-water they cease producing fertilizin within two hours after five or six washings. If eggs be washed by removal directly to a large quantity of sea-water the residual fertilizin (in the jelly which may not be removed by butyric acid) is washed away so that when tested these eggs are negative; while an equal bulk of unfertilized eggs *without* jelly suspended in a like amount of sea-water will still give the reaction.

We may conclude that after membrane formation induced by sperm or by butyric acid eggs no longer secrete fertilizin.

According to Lillie's studies, and likewise Moore's account ('16), fertilization capacity and fertilizin production in *Arbacia* run parallel. I have found the same to be true in *Platynereis* and *Nereis*. Farther, *Nereis* exhibits a very striking phenomenon for even slight washing renders impossible initiation of development by warming. In the eggs of *Echinarachnius* the measure of fertilizability is agglutinin production.

C. *Summary of Part II.*

We have shown (1) that *Echinarachnius* eggs in sea-water liberate a substance that agglutinates *Echinarachnius* sperm. This substance, fertilizin, which can be quantitatively studied is formed most abundantly in fully ripened (shed) eggs. The substance is lost by eggs repeatedly washed. It is formed by ripe eggs with or without jelly but is not formed by immature eggs nor is it found in perivisceral fluid. (2). Fertilizin production is an index of fertilization capacity. Eggs highest in fertilizin content, shed eggs, fertilize in largest numbers. Washing the eggs decreases both the fertilizin secretion and the percentage of fertilization. Eggs normally resistant to fertilization yield little fertilizin. After membrane formation fertilizin production ceases.

III. Cross Fertilization and Agglutination in *Echinarachnius* and *Arbacia*.

We have seen in Part II. that the eggs of *Echinarachnius* in sea-water liberate a substance comparable in every way to the substance, fertilizin, liberated by *Arbacia* eggs. I have, therefore, used the term fertilizin for the *Echinarachnius* substance. The problem that next presented itself was the study of cross-fertilization in these two echinids whose sperm behave in the same way to their egg secretions. In a relatively short time, a rather curious, but as it turned out merely apparent, contradiction was in evidence: The cross was highly successful in one direction only and the agglutination reaction ran counter to and not parallel with cross fertilization capacity. This seemed indeed worthy of study. A great deal of time, therefore, was devoted to repeating the initial attempts at cross fertilization and to determining the nature of, and the part played by, the hetero-agglutinating substance.[1]

In this section I shall present first the results of the cross fertilization and second some experiments which attempt to analyze the significance of the hetero-agglutination.

A. *Reciprocal Cross Fertilization of Echinarachnius and Arbacia.*

With various methods many echinid crosses have been obtained by a large number of workers; of these, the crosses by Tennent and by Vernon may be particularly mentioned because of the widely differing forms employed. The value of these methods is discussed by Tennent and need not be commented on here. Moreover, my object was not so much to test the relative value of these methods for *Arbacia-Echinarachnius* crosses as to test under the same conditions the response of the *Arbacia* egg and of the *Echinarachnius* egg to foreign sperm. Comparison of the capacity of the eggs for cross fertilization is made only where the same method was employed on both eggs. And I may say at once that while it is relatively easy to fertilize *Echinarachnius* eggs with *Arbacia* sperm, the reciprocal fertilization is extremely difficult.

[1] Abundant material of both straight and cross fertilizations was fixed in a number of fluids. The results of the study of this will appear later.

Three methods were used to obtain cross fertilization: allowing the eggs to stand, treating them with alkali, and subjecting them to concentrated sperm. In each experiment reciprocal crosses[3] were made, but it will be convenient to consider them separately. I shall begin with the *Arbacia* ♀ × *Echinarachnius* ♂.

1. *Arbacia* ♀ × *Echinarachnius* ♂.

1. *Arbacia eggs allowed to stand* in sea-water upward to thirty-six hours show little development following insemination with *Echinarachnius* sperm. For example, on July 2 I made an observation subsequently repeated throughout the season:

July 2. Eggs of *Arbacia* placed in a finger bowl of sea-water from which two samples were removed at hour intervals, from 8 A.M. to 10 P.M.; one sample inseminated with *Echinarachnius* sperm, the other uninseminated control. Hourly inseminations were continued during the next day until 4:00 P.M., a final insemination at 6:00 P.M. One lot, 24-hour, showed 1 per cent. cleavage, its control about 0.1 per cent.

Results with shed eggs placed in sea-water without subsequent washing and with eggs thoroughly washed by changing the water repeatedly show slight differences.

2. *With alkali treatment* the results are practically negative for the amounts of alkali used; whenever development followed the per cent. was extremely low.

July 7, 5:00 P.M. Eggs in 10 c.c. of sea-water plus 2 drops of *n*/10 KOH were inseminated with *Echinarachnius* sperm. 6:40 P.M. Not a single egg formed a membrane. Later none of these developed.

3. *Treatment with concentrated sperm* gave the best results between one and three per cent. of eggs developing. For example, I quote from a long experiment of July 9.

5:00 PM. Five drops of dry eggs from one *Arbacia* were put in each of four watch glasses *A*, *B*, *C*, *D*. To *A*, one drop of *Echinarachnius* sperm suspension added and 5 c.c. of sea-water; to *B*, one drop of *Echinarachnius* sperm suspension and after five minutes 5 c.c. of sea-water; to *C Arbacia* sperm and 5 c.c. of sea-water; *D*, uninseminated control. *A* gave 1 per cent. cleavage; *B* 2 per cent.; *C*, 95 per cent.; and *D* not *one membrane or a cleavage.*

Washing the eggs thoroughly would seem to be beneficial to treatment with concentrated sperm but the data on this I deem at present insufficient to be conclusive.

On the whole, the cross, *Arbacia* ♀ × *Echinarachnius* ♂ *is*

not highly successful; often none of the methods gave results. The reciprocal is not so difficult.

2. *Echinarachnius* ♀ × *Arbacia* ♂.

The cross *Echinarachnius* ♀ × *Arbacia* ♂ as well as the reciprocal cross was made usually during agglutination experiments. Thus, after removing the supernatant sea-water from eggs for agglutination tests these could be used for straight or cross fertilization. This method, in addition to being economical, made possible the performing of a large number of experiments during the time the *Echinarachnius* eggs are at their best.

1. *Allowing the Eggs to Stand.*—Although as mentioned above, *Echinarachnius* eggs are not so resistant to sea-water as *Arbacia* eggs, after three to four hours in sea-water they develop following insemination with *Arbacia* sperm. Thus on July 4, *Echinarachnius* eggs were inseminated with *Arbacia* sperm at ten-minute intervals up to three hours. The best lot of eggs was that inseminated after two hours in sea-water and showed five per cent. of development. Addition of *Arbacia* egg-water to the eggs does not increase the per cent. of those that develop.

2. *Treating the Eggs with Alkali.*—Treatment with alkali before and after insemination gives a high per cent. of cleavage; the eggs, however, in the later stages, particularly the blastula and gastrula, show marked abnormalities as well as a tendency to disintegrate.

On July 7, the following experiment was made three times:

2:40 P.M. Eggs previously procured by straining from ovaries gently cut up in sea-water were divided among seven dishes:

No. 1, uninseminated control in 15 c.c. of sea-water.

No. 2, eggs + 15 c.c. sea-water + 1 drop *n*/10 KOH.

No. 3, eggs + 15 c.c. sea-water + 2 drops *n*/10 KOH.

No. 4, eggs + 15 c.c. sea-water + 3 drops *n*/10 KOH.

2:42 P.M. Nos. 2, 3 and 4 inseminated with freshly made thin *Arbacia* sperm suspension.

2:45 P.M. Nos. 5, 6 and 7. Eggs in 15 c.c. of sea-water inseminated with same *Arbacia* sperm.

2:47 P.M. 1, 2 and 3 drops *n*/10 KOH added to nos. 5, 6, and 7 respectively.

5:00 P.M. Eggs show five per cent. development; in No. 2, much less in 3, 4, 5, 6, 7. Control shows no development.

With shed eggs the results are strikingly different.

July 8, 3:10 P.M. To dry eggs from one female, 100 c.c. sea-water added.

3:20 P.M. To each of seven watch glasses a drop of eggs is added.

No. 1, uninseminated control in 15 c.c. of sea-water.

No. 2, Eggs + 15 c.c. of sea-water + 1 drop *n*/10 KOH.

No. 3, Eggs + 15 c.c. of sea-water + 2 drops *n*/10 KOH.

No. 4, Eggs + 15 c.c. of sea-water + 3 drops *n*/10 KOH.

3:22, Nos. 2–4 inseminated each with one drop of freshly prepared active *Arbacia* sperm suspension.

3:24. Nos. 5, 6, and 7, each with 15 c.c. of sea-water inseminated with one drop of same *Arbacia* sperm.

3:26. To nos. 5, 6 and 7 respectively are added 1, 2 and 3 drops of *n*/10 KOH.

5:00. These eggs show high per cent. cleavage (90 per cent. in no. 4), but also a tendency to lose their membranes when put in normal sea-water. Control, no development.

3. *Insemination with Excess of Sperm.*—The method of heavy insemination succeeds with *Echinarachnius* eggs, giving ten to twenty-five per cent. of cleavage. The later stages are far superior to those eggs in which crossing has been induced through alkali treatment.

July 3, 9:10 A.M. Eggs removed from ovaries divided into three lots—*A*, *B* and *C*. *Lot A* inseminated at 9:15 A.M. with heavy *Arbacia* sperm; *Lot B* similarly at 11:00 A.M.; and *Lot C* similarly at 2:55 P.M. *Lot A* shows 13 per cent. of development at 2:50. *Lot B* 8 per cent. *Lot C* at 5:00 P.M. shows no development.

July 11, 9:30 A.M. 1/2 c.c. of dry *Echinarachnius* eggs placed in 10 c.c. of sea-water. 10:05 A.M., five drops of eggs in each of four watch glasses (Series I.) containing 1, 2, 3 and 4 drops of freshly prepared *Arbacia* suspension. To each of four watch glasses (Series II.) containing five drops of eggs, 1, 2, 3 and 4 drops of *Arbacia* sperm added. Watch glass no. 9 uninseminated control; no. 10, inseminated with *Echinarachnius* sperm.

1:30 P.M. Uninseminated control, no development. Eggs inseminated with *Echinarachnius* sperm 98 per cent. developing. Eggs of both series inseminated with *Arbacia* sperm developing—25 per cent. in most dishes.

These experiments with heavy insemination show that the eggs develop best when inseminated soon after procured; also, that dry eggs are better than "ovary eggs." Whether eggs are added to sperm or vice versa is of no monent, but sperm must be fresh. The eggs treated with dense sperm suspension develop more nearly like the straight fertilized eggs than either the alkali treated or the stale eggs. The larvæ are vigorous and active, swimming to the top as do normal larvæ.[1]

The cross *Echinarachnius* ♀ × *Arbacia* ♂ succeeds far better than the *Arbacia* ♀ × *Echinarachnius* ♂.[2] Indeed in some cases the *Arbacia* egg is in no wise affected by *Echinarachnius*

[1] Larvæ from this cross were kept for four weeks.

[2] Cf. Gemmil's results with the cross, *Cribrella* × *Asterias*.

sperm. Thus, we have a sharply defined difference in behavior between the *Echinarachnius* egg and the *Arbacia* egg with respect to foreign sperm.

B. *Hetero-agglutination in Echinarachnius and Arbacia.*

If fertilizin is necessary for fertilization and if it is likewise specific, we should expect that where cross fertilization is possible in one direction only the sperm agglutination reaction would, if it occurred at all, take place in the same direction The opposite appears to be the case for the *Echinarachnius* × *Arbacia* cross since *Echinarachnius* egg-water has no effect on *Arbacia* sperm while *Arbacia* egg-water has a powerful agglutinating effect on *Echinarachnius* sperm. However, as shown beyond this is not the effect of *Arbacia* sperm agglutinin but is due to a separate substance, a hetero-agglutinin.

1. *Effect of Echinarachnius Egg-water on Arbacia Sperm.*

Sea-water from above *Echinarachnius* eggs highly charged with the substance that agglutinates *Echinarachnius* sperm has no agglutinative action on *Arbacia* sperm. This is true of the most powerful *Echinarachnius* egg-water that was used. The *Echinarachnius* egg-water does, however, intensely activate *Arbacia* sperm.

Experiments were made with egg-water of both " ovary " and dry eggs.

July 2, 10:00 A.M. Eggs from four *Echinarachnius* strained and washed 5 times in 250 c.c. of sea-water. 2 c.c. of eggs and small bits of ovary. 12:00 M., 210 c.c. of sea-water removed and filtered. Tests with *Echinarachnius* sperm, at 1:30 P.M. and 8:30 P.M., showed great agglutinating strength.

11:00 A.M. Eggs from *Arbacia* washed 5 times in 100 c.c. of sea-water at each washing. 12:05 P.M., 45 c.c. of supernatant sea-water removed and filtered. Tested with *Arbacia* sperm, showed very powerful reaction.

3:00 P.M. *Arbacia* egg-water agglutinates *Arbacia* sperm; *Echinarachnius* egg-water agglutinates *Echinarachnius* sperm, but fails to agglutinate *Arbacia* sperm—trials being made on sperm of three males.

July 10, 10:30 A.M. 1 1/2 c.c. of dry eggs of *Echinarachnius* in 8 1/2 c.c. of sea-water. 11:00 A.M. 8 c.c. of sea-water were pipetted off and filtered. (8 c.c. of fresh sea-water added to the eggs.) 11:05 A.M. 7 c.c. of *Arbacia* eggs from four females put in 50 c.c. of sea-water.

12:10 P.M. *Echinarachnius* secretion gives a 5–6-second reaction with its sperm on 1/500 dilution; full strength, this fails to agglutinate *Arbacia* sperm. *Arbacia* sperm suspensions used were 50 per cent., 20 per cent., 10 per cent. and 1 per cent. freshly prepared in each case. *Arbacia* egg-water agglutinates *Arbacia* sperm.

2:15 P.M. 8 c.c. of sea-water pipetted from the *Echinarachnius* eggs and filtered (8 c.c. of sea-water added to the eggs). 2:00 to 4:00 P.M. This egg-water removed (second washing) gave on *Echinarachnius* sperm a 16-second reaction at 1/12,800 dilution. It failed to agglutinate *Arbacia* sperm but activated them very intensely. *Arbacia* egg-water on these same sperm gave an 8-second reaction on 1/6,400 dilution.

July 16. A strong *Echinarachnius* egg-water, 1/12,800 power, tested on *Arbacia* sperm gave no agglutination, but very intense activation.

The experiments here cited represent but a small percentage of the total number made with *Echinarachnius* egg-water on *Arbacia* sperm. There can thus be no question as to the failure of the *Echinarachnius* egg-water to agglutinate *Arbacia* sperm. On the other hand, the stimulation to increased motility induced by *Echinarachnius* egg-water is very remarkable.[1] In no other case that I know is the activation so marked. It will be noted that the egg-water used is that charged either by thoroughly washed "ovary eggs" or by dry eggs; this, a fact which may be significant, must mean that such sea-water contains very little if any perivisceral fluid.

2. *Effect of Arbacia Secretion on Echinarachnius Sperm.*

Sea-water charged by *Arbacia* eggs with an agglutinating substance for *Arbacia* sperm likewise contains a substance that agglutinates *Echinarachnius* sperm. Certain characters of this agglutination of *Echinarachnius* sperm show that it is due to a hetero-agglutinin distinct from the *Arbacia* sperm agglutinin.

(*a*) *Agglutinative Power of Arbacia Egg-water on Echinarachnius Sperm after Successive Dilution.*—In the first place it may be noted that on dilution of *Arbacia* egg-water the agglutinating substance for *Echinarachnius* sperm drops out before the agglutinin for *Arbacia* sperm. A freshly prepared suspension made of unwashed "ovary eggs" may agglutinate *Echinarachnius* sperm in a very powerful manner, irreversibly so in some cases; but if the egg-water stand or be diluted this effect will gradually disappear, the iso-agglutinin remaining. On the other hand, a suspension of shed eggs not uncommonly gives no agglutinin for *Echinarachnius* sperm, though this class of eggs most actively secrete iso-agglutinin.

[1] *Asterias* sperm exhibit an interesting phenomenon. When a fresh suspension is prepared, the sperm may be absolutely immobile and remain thus. If now they be mixed with eggs inseminated within one or two minutes previously, they are stimulated to great activity so that they may roll the eggs around.

July 10, 11:05 A.M. 7 c.c. of *Arbacia* eggs from four females in 50 c.c. of sea-water. 12:15, 40 c.c. pipetted off and filtered (40 c.c. of fresh sea-water added to the eggs). 4:00 P.M. *Arbacia* egg-water (first washing) gave an 8-second reaction with *Arbacia* sperm at 1/12,800 dilution. This same suspension gave a 3–4-second reaction on *Echinarachnius* sperm at 1/800 dilution.

July 16, 11:00 A.M. 7 c.c. of *Arbacia* eggs and ovarian tissue put in 50 c.c. of sea-water; strained giving 55 c.c. 1:40 P.M. 37 c.c. of supernatant sea-water pipetted off and filtered, 80 c.c. of sea-water being added to eggs. 2:30 P.M. Supernatant sea-water withdrawn gave at 1/6,400 dilution a 6-second reaction with *Arbacia* sperm; faintly positive for *Echinarachnius* at 1/100 dilution.

(*b*) *Hetero-agglutinin Production of Washed Arbacia Eggs.*—In the second place, if *Arbacia* eggs be repeatedly washed the hetero-agglutinating substance drops out before the iso-agglutinin. A single washing may suffice to remove the hetero-agglutinin. Certainly washing during twelve hours is sufficient entirely to remove the hetero-agglutinin.

July 12, 6:30 P.M. *Arbacia* eggs in 250 c.c. of sea-water; eggs settle at the 7-c.c. mark. 140 c.c. poured off and 140 c.c. sea-water added.

7:30 P.M.—220 c.c. sea-water poured off, 220 c.c. added.
8:30 P.M.—190 c.c. sea-water poured off, 190 c.c. added.
10:00 P.M.—140 c.c. sea-water poured off, 140 c.c. added.
July 13.
8:15 A.M.—210 c.c. sea-water poured off, 210 c.c. added.
9:15 A.M.—150 c.c. sea-water poured off, 150 c.c. added.
10:10 A.M.—200 c.c. sea-water poured off, 200 c.c. added.
11:45 A.M.—240 c.c. sea-water poured off, 240 c.c. added.

At 11:45 A.M. 2 c.c. of eggs left. At 12:30 P.M. this supernatant sea-water gave a 6-second reaction at 1/100 dilution with *Arbacia* sperm. With *Echinarachnius* sperm the last positive reaction made with undiluted supernatant sea-water was at 9:50 P.M., July 12. 8:05 A.M., July 13, test was negative with *Echinarachnius* sperm.

The eggs used July 16 in the experiment cited above were farther washed as follows: 2:35, 3:06 and 5:15 P.M. by removing 90, 94 and 93 c.c. of sea-water, replacing in each case an amount equal to that withdrawn. The last positive effect with undiluted egg-water on *Echinarachnius* sperm was at 3:06 P.M. At 5:15 P.M. the last washing gave a positive reaction with *Arbacia* at 1/1,600 dilution.

In these washing experiments because eggs are lost, it might be argued that the hetero-agglutinin disappears because of the insufficient number of eggs left to charge the sea-water. But since the activity of the hetero-agglutinin in any case is inconstant, its loss in no wise paralleling that of the iso-agglutinin, this argument is scarcely tenable. Nothing is surer than the variation in hetero-agglutinin production by equal bulks of eggs from zero upward.

(*c*) *Precipitation of the Hetero-agglutinin by Echinarachnius Sperm.*—Thirdly, it can be shown that the agglutinin for *Echinarachnius* sperm present in sea-water above *Arbacia* eggs can be precipitated by *Echinarachnius* sperm leaving the *Arbacia* sperm agglutinin practically undiminished in strength. Thus, on July 15, I prepared three vials each with 1 c.c. of filtered *Arbacia* egg-water; to these vials, *A*, *B*, and *C* respectively were added 1, 2 and 4 drops of *Echinarachnius* sperm suspension. A fourth vial, *D* (control), contained 5 c.c. of *Arbacia* egg-water with no sperm. After five minutes the following results on *Echinarachnius* sperm were recorded: *A*, powerful agglutinant undiluted; negative at 1/8 dilution; *B*, positive; *C*, positive.

After ten minutes, *C* was negative; after fifteen *B* faintly positive; at twenty minutes all vials were negative.

Tested on *Arbacia* sperm, the vials *A*, *B* and *D* (control) gave a 6-second reaction at 1/400 dilution.

This experiment when repeated gave essentially the same results: the *Echinarachnius* sperm agglutinin may be completely removed, although this removal is not immediate, the rapidity with which the hetero-agglutinin is destroyed depending upon the density of the *Echinarachnius* sperm suspension used; the iso-agglutinin, on the other hand, remains undiminished in power.

(*d*) *Agglutinative Action of Arbacia Blood on Echinarachnius Sperm.*—Farther, it can be demonstrated that the perivisceral fluid of *Arbacia* has an agglutinative action on *Echinarachnius* sperm though lacking this action on *Arbacia* sperm. Thus on July 17, I carefully collected blood from four *Arbacia* which after clotting was filtered. Ten determinations on *Arbacia* sperm were negative in each case, the sperm showing, however, undiminished response to *Arbacia* egg-water tested after each trial with the blood. This perivisceral fluid agglutinated sperm from six *Echinarachnius* tested in turn.

Frequently, as in another observation on this same day, July 17, females were found whose blood was negative to both *Arbacia* and *Echinarachnius* sperm. Blood is positive with *Arbacia* sperm if the ovaries are ruptured when the animals are opened. Extreme care is necessary to avoid this.

(*e*) *Microscopic Appearance of Echinarachnius Sperm Agglutinated by Arbacia Egg-water.*—Finally, it may be noted that the microscopic appearance of *Echinarachnius* sperm agglutination by *Arbacia* egg-water is markedly different from agglutination by *Echinarachnius* egg-water. On this point I am borne out by four investigators who were good enough from time to time to observe the phenomena. In every instance they declared that they could note a difference in the character of the agglutination. When *Echinarachnius* egg-water is blown into a drop of *Echinarachnius* sperm suspension, the agglutination is sharply defined because after the initial activation the spermatozoa clump, leaving spaces between the clumps practictically free of sperm. After a time, depending upon the strength of the egg-water the clumps break up, the individual sperm being again homogeneously distributed throughout the field. With *Arbacia* egg-water the sperm of *Echinarachnius* behave differently. The agglutinated masses tend to string out with some free sperm among them. The agglutination is slower in beginning and with strong egg-water slow in breaking up, indeed may never break up, the agglutination being permanent. The whole picture suggests a coagulative effect. Agglutination of *Echinarachnius* sperm by *Arbacia* egg-water probably is much like that of *Nereis* sperm by *Arbacia;* this point I attempted to determine but could not succeed in getting *Nereis* sperm at the proper time.

The evidence here presented shows that the iso-agglutinin and hetero-agglutinin found in *Arbacia* egg-water are separate. What the source is, one can scarcely say, although it may well be the blood. Suspensions of eggs thoroughly washed free of perivisceral fluid would, therefore, fail to charge sea-water with the hetero-agglutinin, while the eggs would continue actively to produce the iso-agglutinin. Whatever, the source of the substance, the experiments cited above indicate that it is distinct from the iso-agglutinin and is of the nature of a toxic substance.

C. *Summary of Part III.*

Summarizing we find (1) that cross fertilization between *Echinarachnius* and *Arbacia* is possible but that the *Arbacia* egg

is greatly resistant to *Echinarachnius* sperm while the *Echinarachnius* egg is easily fertilized by *Arbacia* sperm. (2) *Echinarachnius* egg-water activates but does not agglutinate *Arbacia* sperm. *Arbacia* egg-water agglutinates *Echinarachnius* sperm. This is a hetero-agglutination by a substance in *Arbacia* egg-water separate from *Arbacia* fertilizin because it may be removed from the egg-water by dilution, by repeatedly washing the eggs and by precipitating it with *Echinarachnius* sperm. It is found in *Arbacia* blood. The microscopic appearance of this toxic hetero-agglutination of *Echinarachnius* sperm is different from that of the iso-agglutination.

IV. Discussion.

Every egg has a fertilizable period during which falls its optimum capacity for fertilization. Thus, *Asterias* eggs normally shed after complete maturation are capable of fertilization on immediate insemination but if procured by placing the ovaries in sea-water these eggs, in the germinal vesicle stage, fertilize only after the maturation process. Apparently, this is true of *Chætopterus* and *Cerebratulus* eggs which come into sea-water in the germinal vesicle stage: insemination is most effective after the germinal vesicle fades and the first maturation spindle is at the mesophase.[1] Immature eggs will not fertilize though sperm may bore into such eggs. Thus, in preparations of the normal fertilization of *Arbacia*, I find many ovocytes with one or more sperm in the cytoplasm, in some cases around the germinal vesicles. Such sperm undergo no change. Moreover, sperm may normally enter immature eggs according to Hempelmann, Shearer and von Hofsten. Sperm entry, therefore, is no criterion of fertilization. Moreover, the fertilizable period of an egg gradually passes over as can be shown by the process of staling, allowing the eggs to stand in normal sea-water. This may take place as in *Platynereis* in an almost incredibly short time; in *Echinarachnius* the time before capacity for fertilization is lost is much longer though not so long as in *Arbacia*. Sperm may

[1] Wilson has found that in *Cerebratulus* after the germinal vesicle has broken down, merogony is possible; enucleated fragments after insemination are not capable of refertilization. (Cf. also Delage.)

enter these stale eggs likewise; for example, in *Platynereis* (Just, '15*b*) sperm not only enter but come into apposition with the female nucleus without inducing development though maturation has been complete. Through the work of Lillie on *Arbacia* these phenomena may be interpreted: immature eggs, stale eggs and fertilized eggs produce no fertilizin; they are incapable of fertilization. One cannot help but postulate a causal connection. Much the same interpretation is possible in *Nereis* and *Platynereis*. In *Echinarachnius* the process is in every way similar to that in *Arbacia*. Whatever prejudice one may have to the fertilizin theory of fertilization because of the immunological terminology, the proved facts one must admit. At the very least, fertilizin is an index to fertilization capacity.

Again, eggs show a period during which they are most susceptible to agents that initiate development. This has been most clearly shown for the starfish by Mathews and by R. S. Lillie. It is generally known that the eggs of some females respond more readily to agents that initiate development than others. In the *Nereis* (Just, '15*a*) one may observe a very striking behavior of the egg to warmed sea-water. From a female quickly dried, dry eggs are taken and divided into two lots. One lot is placed in a small quantity of sea-water at 33° C. and the other into ordinary sea-water. Those eggs in warm sea-water will form jelly, maturate and cleave, the resulting embryos closely simulating the normal. If the eggs in ordinary sea-water be transferred to warmed sea-water *they do not even form jelly*. Nothing is more striking than the comparison between two lots of *Nereis* eggs that have been subjected to warming with and without previous exposure to ordinary sea-water: the dry eggs are in a thick mat of jelly, the washed eggs closely crowded together without any jelly formed. In *Nereis*, then, the egg must have its full content of fertilizin in order to respond to warming as a means of artificial parthenogenesis: it loses its power for initiation of development by artificial means before it loses its fertilizing power. Muscle, nerve and gland respond to artificial stimulation, but the normal mode is most effective. So the sperm is the most efficient activator for the egg. In *Nereis* the best time for artificial parthenogenesis is the time of greatest fertilizin content.

Farthermore, the studies of Lillie have shown that like the fertilized egg, the egg induced to development artificially no longer produces fertilizin. Moore's ('16, '17) studies confirm and extend those of Lillie.

The presence of fertilizin in *Echinarachnius*, *Arbacia* and *Nereis* is indicated by its power to agglutinate specific sperm. But this very agglutination of the sperm is held as an argument against the fertilizin theory because it is held to "inhibit the fertilizing effect of the spermatozoa instead of enhancing it since the cluster formation prevents the sperm from reaching the egg. Even from a teleological viewpoint it is difficult to understand why a substance which only prevents the fertilizing action of the sperm should be a necessary link in such action" (Loeb, '14). First, however, it should be recalled that this agglutination is a reversible process where specific sperm are employed, the clumping lasting from ten to twenty seconds for average egg-water dilution. Second, while Lillie found that agglutination reduces the per cent. of fertilization, Fuchs observed the contrary to be the case. Finally, we should note these agglutinations are made in the laboratory with fairly thick sperm suspension and that not only the motility of the sperm but the density of the suspension are functions in the agglutination reaction. Agglutination would scarcely take place in the sea where the conditions are not those in a drop of water on a slide. As a matter of fact agglutination rarely takes place during insemination in a finger-bowl because we very carefully use thin sperm suspensions to avoid polyspermy. The sperm are thus too widely separated to clump; instead, they stick to the eggs. I, therefore, heartly agree with Loeb when he states that he is "inclined to believe that the cluster formation or agglutination of sperm does not occur when fertilization takes place under natural conditions." But this in no wise invalidates the claims of the fertilizin theory.

Concerning the sperm agglutination by egg-water of another form, hetero-agglutination, it seems to me that it would be necessary to know the *nature* of the agglutination; this demands the microscopic method of studying the agglutination. Thus, *Arbacia* egg-water agglutinates *Nereis* sperm, but this is a toxic

agglutination produced by a substance distinct from the iso-agglutinin; it is found in *Arbacia* blood. Likewise the agglutinin in *Arbacia* egg-water for *Echinarachnius* sperm is separate from the iso-agglutinin. These points could be better determined by the microscopic method, by examining the agglutination after diluting the egg-water, attenuating the agglutinin by washing the eggs, etc. The macroscopic method of studying the agglutination would fail. It may be, farthermore, that *Arbacia* blood is generally toxic. Thus, I have found that it will induce jelly-formation, maturation, and if allowed to act too long cytolysis in the *Nereis* egg. We may readily understand that when cross fertilization is possible between forms in one direction only the fact that an agglutination takes place in the opposite direction does not invalidate the fertilizin theory of fertilization if the agglutination is a toxic hetero-agglutination.

Finally, the activation of sperm by egg-water may be of great importance in fertilization as Loeb ('15) points out. Thus, *Echinarachnius* egg-water activates *Arbacia* sperm which are capable of fertilizing *Echinarachnius* eggs. In *Asterias* if at the moment of insemination by immobile sperm more sperm be added they are greatly activated. In *Nereis* agglutination is only possible with very active sperm; such sperm spontaneously form aggregations when mixed with sea-water alone. Sperm that do not form these spontaneous aggregations will not agglutinate. Also in both *Arbacia* and *Echinarachnius* the first stage in the agglutination reaction is activation. Probably in those *Echinarachnius* eggs normally resistant to fertilization there exists a membrane condition that masks the presence of fertilizin. In addition, therefore, to the farther study of activation we must approach the investigation of just such conditions. By these analyses the fertilizin theory may prove of wider application.

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